**PCT** WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : C12N 5/00, 5/02, 11/00 — A1

(11) International Publication Number: **WO 98/04680**

(43) International Publication Date: 5 February 1998 (05.02.98)

(21) International Application Number: PCT/US97/13079

(22) International Filing Date: 25 July 1997 (25.07.97)

(30) Priority Data:
60/022,661 26 July 1996 (26.07.96) US

(71) Applicant *(for all designated States except US)*: UNIVERSITY OF MANITOBA [CA/CA]; Administration Building, Room 202, Winnipeg, Manitoba R3T 2W2 (CA).

(71) Applicant *(for MW only)*: KOHN, Kenneth, I. [US/US]; Suite 410, 30500 Northwestern Highway, Farmington Hills, MI 48334 (US).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: BUTLER, Michael [CA/CA]; 369 Montrose Street, Winnipeg, Manitoba R3M 3M1 (CA). BERRY, John, Michael [CA/CA]; 19 Prestwood Court, Maple, Ontario L6A M9 (CA).

(74) Agents: MONTGOMERY, Ilene, N. et al.; Kohn & Associates, Suite 410, 30500 Northwestern Highway, Farmington Hills, MI 48334 (US).

(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, HU, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).

**Published**
*With international search report.*

(54) Title: SERUM-FREE MEDIUM FOR GROWTH OF ANCHORAGE-DEPENDANT MAMMALIAN CELLS

(57) Abstract

A serum-free medium for growing anchorage-dependent mammalian cells in tissue culture including a basal medium supplemented with components for replacing serum at the concentration range as set forth in Table 1 is disclosed. The tissue culture is stationary or agitated using microcarriers. In an embodiment, the fibronectin is added to the medium immediately prior to use. Alternatively, the fibronectin is used to precoat tissue culture flasks or the microcarriers. In an embodiment, the present invention provides a serum-free medium for growing anchorage-dependent Vero (African green monkey) cells in tissue culture including a basal medium supplemented with the components at the concentration range as set forth in Table 2. The present invention also provides a method of culturing anchorage-dependent mammalian cells, such as Vero cells, in tissue culture including the step of culturing in a basal medium supplemented with the components at the concentration range as set forth in Table 1.

*FOR THE PURPOSES OF INFORMATION ONLY*

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| **AL** | Albania | **ES** | Spain | **LS** | Lesotho | **SI** | Slovenia |
| **AM** | Armenia | **FI** | Finland | **LT** | Lithuania | **SK** | Slovakia |
| **AT** | Austria | **FR** | France | **LU** | Luxembourg | **SN** | Senegal |
| **AU** | Australia | **GA** | Gabon | **LV** | Latvia | **SZ** | Swaziland |
| **AZ** | Azerbaijan | **GB** | United Kingdom | **MC** | Monaco | **TD** | Chad |
| **BA** | Bosnia and Herzegovina | **GE** | Georgia | **MD** | Republic of Moldova | **TG** | Togo |
| **BB** | Barbados | **GH** | Ghana | **MG** | Madagascar | **TJ** | Tajikistan |
| **BE** | Belgium | **GN** | Guinea | **MK** | The former Yugoslav Republic of Macedonia | **TM** | Turkmenistan |
| **BF** | Burkina Faso | **GR** | Greece | | | **TR** | Turkey |
| **BG** | Bulgaria | **HU** | Hungary | **ML** | Mali | **TT** | Trinidad and Tobago |
| **BJ** | Benin | **IE** | Ireland | **MN** | Mongolia | **UA** | Ukraine |
| **BR** | Brazil | **IL** | Israel | **MR** | Mauritania | **UG** | Uganda |
| **BY** | Belarus | **IS** | Iceland | **MW** | Malawi | **US** | United States of America |
| **CA** | Canada | **IT** | Italy | **MX** | Mexico | **UZ** | Uzbekistan |
| **CF** | Central African Republic | **JP** | Japan | **NE** | Niger | **VN** | Viet Nam |
| **CG** | Congo | **KE** | Kenya | **NL** | Netherlands | **YU** | Yugoslavia |
| **CH** | Switzerland | **KG** | Kyrgyzstan | **NO** | Norway | **ZW** | Zimbabwe |
| **CI** | Côte d'Ivoire | **KP** | Democratic People's Republic of Korea | **NZ** | New Zealand | | |
| **CM** | Cameroon | | | **PL** | Poland | | |
| **CN** | China | **KR** | Republic of Korea | **PT** | Portugal | | |
| **CU** | Cuba | **KZ** | Kazakstan | **RO** | Romania | | |
| **CZ** | Czech Republic | **LC** | Saint Lucia | **RU** | Russian Federation | | |
| **DE** | Germany | **LI** | Liechtenstein | **SD** | Sudan | | |
| **DK** | Denmark | **LK** | Sri Lanka | **SE** | Sweden | | |
| **EE** | Estonia | **LR** | Liberia | **SG** | Singapore | | |

# SERUM-FREE MEDIUM FOR GROWTH OF ANCHORAGE-DEPENDENT MAMMALIAN CELLS

## FIELD OF THE INVENTION

The present invention provides a serum-free cell culture medium for anchorage-dependent mammalian cells.

## BACKGROUND OF THE INVENTION

Mammalian cells are used in large-scale bioprocesses for the production of health-care products such as vaccines and recombinant proteins that are the basis of the commercial operations of many biotechnology and pharmaceutical companies. The cells are grown in a liquid medium of defined chemicals which is often supplemented with blood serum, typically at 10% v/v. The serum is normally of bovine origin and provides undefined hormones, growth factors and micronutrients required for cell growth.

However there are a number of disadvantages of using serum in these processes: (a) The batch to batch variation in the composition of serum causes variability in cell growth. (b) The high protein content causes difficulties in product purification. (c) There is an unacceptable risk of viral or mycoplasma contamination. Serum of bovine origin may also be contaminated by the undefined agent of bovine spongiform encephalopathy ("mad cow disease") with associated risks to humans. (d) Fetal bovine serum which is the primary choice for some processes is expensive and subject to periodic world shortages.

These factors outline the importance of developing an effective chemically defined formulation that can substitute for serum in mammalian cell culture processes.

Serum-free media formulations are used routinely for suspension cultures [Butler, 1992]. For some widely used cells that can be grown in suspension such as hybridomas or CHO (chinese hamster ovary), serum-free formulations

containing a cocktail of up to five ingredients have been developed and are now used routinely. However, anchorage-dependent mammalian cells are more fastidious in their growth requirements and serum-free formulations for such cells are not as satisfactory as for suspension cultures. Nevertheless the need for serum-free cultures of anchorage-dependent cells is considerable.

Anchorage-dependent mammalian cell cultures are used exclusively for human viral vaccine production and the generation of artificial skin used in the treatment of human injuries. Also, anchorage-dependent cells are used in many processes for the production of veterinary vaccines and recombinant proteins. Anchorage-dependent cell lines widely used in commercial bioprocesses include: MRC-5, WI-38, FS-4 (human fibroblasts), Vero (African green monkey), BHK (Baby hamster kidney) and MDCK (Madin Darby canine kidney).

Taub et al [1979] made a breakthrough with the development of a serum-free formulation (Medium K-1) which supported adherent MDCK cell growth in static cultures for a limited time period (a month). This medium was later developed for MDCK growth in microcarrier cultures [Crespi et al, 1981; Sayer et al, 1987]. Similarly, a serum-free formulation was developed for BHK-21 cells which allows the cells to be grown in an attachment mode in static cultures [Bradshaw et al, 1983]. Both MDCK and BHK cell lines are used for veterinary vaccine and recombinant protein production.

Vero cells are used in microcarrier processes for the production of human polio and rabies vaccines [Montagnon et al, 1984; van Wezel, 1985]. Alternative cell lines for such processes include human diploid fibroblasts such as MRC-5 which are also grown in microcarrier cultures [Forestall et al, 1992]. These viral production processes are normally dependent upon the use of bovine serum with the attendant problems discussed herein above.

Despite their importance for human vaccine production, very few attempts have been made to develop serum-free formulations for growth of Vero cells. Litwin [1992] developed a serum-free formulation for Vero cells but this required a long adaptation period (20-30 days) and was only suitable for growth

of cell aggregates, a form which is generally regarded as unsatisfactory for viral vaccine production. Recent work by Applicants has shown that in order to prevent cell aggregate formation of Vero cells in microcarrier cultures it is important to ensure a high rate of cell to bead attachment at inoculation [Ng et al, 1996].

Cinatl et al [1992] recognized the importance of the cell-substratum interaction when they developed a protein-free medium which was suitable for growth of Vero cells only in static cultures on a modified plastic surface based on polyvinyl formal. Growth on polystyrene culture plates in this medium was poor. Although the initial protein-free formulation produced low growth rates of Vero cells, they later published the full formulation of an improved medium (PFEK-1) which contained 97 components [Cinatl et al, 1993]. They reported growth rates equivalent to serum-based medium on a PVF surface and the production of human pathogenic viruses.

A more recent attempt to produce a serum-free formulation for Vero cells was published by Merten et al [1994]. Here a proprietary formulation (MDSS2) from AXCELL Biotechnologies was described as capable of supporting Vero cell growth in microcarrier cultures. The cells grew in MDSS2 without adaptation and were capable of producing rabies virus. However, the growth curves shown indicate an extremely high doubling time (80 hours) with maximum cell densities occurring after 12 days. The cultures were reported to require five changes of medium (50%) over this period. Similarly, a high doubling time (38 hours) and a long lag phase (4 days) were reported for a serum-free medium by Zhaolie et al [1996].

It would be useful to have a serum-free medium formulation with fewer than 97 components that can be used for anchorage-dependent growth of these cells on various surfaces including T-flasks different microcarrier types. Further it would be useful to have a serum-free formulation in which cells do not clump in culture and that have a high cell growth rate with a minimal lag phase.

## SUMMARY OF THE INVENTION

According to the present invention, a serum-free medium for growing anchorage-dependent mammalian cells in tissue culture as set forth in Table 1 is disclosed. The serum-free medium comprises a basal medium supplemented with chemically defined components (Table 1) which replace the serum. The tissue culture is stationary or agitated using microcarriers. In an embodiment the fibronectin is added to the medium immediately prior to use. Alternatively, the fibronectin is used to precoat tissue culture flasks or the microcarriers.

In an embodiment the present invention provides a serum-free medium for growing anchorage-dependent Vero (African green monkey) cells in tissue culture including a basal medium enriched with the components replacing serum at the concentration range as set forth in Table 2. The tissue culture is stationary or agitated using microcarriers. In an embodiment the fibronectin is added to the medium immediately prior to use. Alternatively, the fibronectin is used to precoat tissue culture flasks or the microcarriers.

The present invention also provides a method of culturing anchorage-dependent mammalian cells in tissue culture including the step of culturing in a basal medium enriched with the components replacing serum at the concentration range as set forth in Table 1. The tissue culture is stationary or agitated using microcarriers. In an embodiment the fibronectin is added to the medium immediately prior to use. Alternatively, the fibronectin is used to precoat tissue culture flasks or the microcarriers.

In a further embodiment the present invention provides a method of culturing anchorage-dependent Vero (African green monkey) cells in tissue culture including the step of culturing in a basal medium enriched with the components for replacing serum at the concentration range as set forth in Table 2. The tissue culture is stationary or agitated using microcarriers. In an embodiment the fibronectin is added to the medium immediately prior to use. Alternatively, the fibronectin is used to precoat tissue culture flasks or the microcarriers.

## DESCRIPTION OF THE DRAWINGS

Other advantages of the present invention will be readily appreciated as the same becomes better understood by reference to the following detailed description when considered in connection with the accompanying drawings wherein:

FIGURE 1 is a graph showing the effect of multiple passages on Vero cell growth in DMEM + 5.0% v/v sCS (open symbols) and VSFM (closed symbols) in T25 culture flasks. The cells were passaged for either 6 (●), 20 (■), 30 (▲) or 35 (▼) passages in each medium. The cells were inoculated at $0.10 \times 10^6$ cells/ml in 10 ml of either VSFM or DMEM + 5.0% v/v sCS. The flasks were incubated with a 10% v/v $CO_2$ overlay. The points are means of n=2.

FIGURE 2A-B are graphs which show the effect of various serum-free medium formulations on preadapted Vero cell growth in 100 ml spinner flasks on (A) 1.00 g/L Cytodex-1 and (B) 1.70 g/L Cultispher-G. The cells were inoculated at $0.05 \times 10^6$ cells/ml in either DMEM + 5.0% v/v sCS (●), VSFM (■), Gibco serum-free Vero maintenance media II (▲), Gibco serum-free Vero maintenance media III (▼), or Celox TCM supplemented, enriched DMEM (◆). The flasks were stirred at 40 rpm continuously (Cytodex-1) or intermittently for 24 hours then continuously and ramped to 60 rpm after 72 hours (Cultispher-G). The flasks were readjusted daily to pH 7.1 after 72 hours in culture with 1.00 M HCl or 2.00 M NaOH. The points are means of n=2.

FIGURE 3 are graphs showing the effects of bead precoating and medium formulation on Vero cell growth in 100 ml spinner flasks on (A) 1.00 g/L Cytodex-1 and (B) 1.70 g/L Cultispher-G. The cells were inoculated at $0.05 \times 10^6$ cells/ml in either DMEM + 5.0% v/v sCS on uncoated beads (●),

VSFM on uncoated beads (■), VSFM (-Fibronectin on uncoated beads (▲), or VSFM (-Fibronectin on DMEM + 5.0% v/v sCS on precoated beads (▼). The flasks were stirred at 40 rpm continuously (Cytodex-1) or intermittently for 24 hours then continuously and ramped to 60 rpm after 72 hours (Cultispher-G). The flasks were readjusted daily to pH 7.1 after 72 hours in culture with 1.00 M HCl or 2.00 M NaOH. The points are means of n=2.

## DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENT

The present invention provides a serum-free medium (SFM) for growing anchorage-dependent (AD) mammalian cells in tissue culture including a basal medium supplemented with the components at the concentration range as set forth in Table 1. By anchorage-dependent cells are meant cells that must attach to a surface during growth. Examples of such cells are MRC-5, WI-38, FS-4 (human fibroblasts), Vero (African green monkey), BHK (Baby hamster kidney) and MDCK (Madin Darby canine kidney) or other anchorage-dependent cells isolated from mammalian tissue for primary culture.

By tissue culture is meant the culturing/growth of cells or tissue slices in an artificial medium, i.e. *ex vivo*. The cells are cultured using either stationary tissue culture or agitated using microcarriers in tissue culture as is generally known in the art.

The supplements or components for the basal medium of the present invention are listed in Table 1 with the appropriate concentration range for mammalian cells. Any basal medium may be used in the practice of the present invention if it supports growth of anchorage-dependent cells when used with serum, with the components as listed in Table 1 of the present invention substituting for the serum. In one embodiment, Dulbecco's modification of Eagle's medium (DMEM) containing 25mM glucose, 4mM glutamine and 36 mM $NaHCO_3$ is the basal medium which is supplemented with the components listed in Table 1. The supplemented medium can be referred to as AD-SFM.

Concentrated stock solutions (x100) are prepared individually or as mixed component stocks. The mixed component stocks include: trace element salts of Se, Fe, Cu and Zn; biotin and vitamin B12; insulin, transferrin, hydrocortisone and triiodothyronine; glutathionine and 2-mercaptoethanol. All stock solutions are prepared as indicated in the Merck Index (10th edition). They are stored at either -20°C (vitamin B12, biotin, insulin, transferrin, hydrocortisone, FGF, triiodothyronine and prostaglandin E1), +4°C (glutathione, fibronectin and fetuin) or room temperature, 20°C (trace elements and mercaptoethanol).

The appropriate growth factor (GF) is selected based on the cell type being cultured as is known in the art. The GFs can include epidermal growth factor (EGF), platelet-derived growth factor (PDGF) or transforming growth factor (TGF), and fibroblast growth factor (FGF). For example, FGF is most suitable for Vero cells.

The order of addition of the supplements to enriched DMEM from concentrated (x100) stock solutions is chosen such that precipitation of any component is avoided. In one embodiment the order is as follows:

1. Add trace elements (Se, Fe, Zn, Cu, Mn, Mo Ni, Si, Sn, V) as the salts listed in Table 1.
2. Add insulin, choline chloride and transferrin.
3. Add glutathione, 2-mercaptoethanol, vitamin B12 and biotin.
4. Sterilize medium by filtration.
5. Add sterile fetuin.
6. Add sterile growth factor (GF), hormones (hydrocortisone and triiodothyronine) and prostaglandin E1.
7. Add fibronectin to the medium immediately prior to use.

In an embodiment, the fibronectin is used to precoat tissue culture flasks or the microcarriers prior to use and fibronectin is not added directly to the medium.

In an embodiment of the present invention, a serum-free medium for growing anchorage-dependent Vero (African green monkey) cells in tissue

culture including a basal medium enriched with the components and concentration range as set forth in Table 2. The formulation in Table 2 is optimal for the growth of Vero cells. The medium in this embodiment is designated VSFM.

The present invention further provides a method of culturing anchorage-dependent mammalian cells in tissue culture including the step of culturing in a basal medium enriched with the components at the concentration range as set forth in Table 1. In a preferred embodiment the cells are Vero cells and the component concentrations are as set forth in Table 2.

The present invention therefore provides a combination of individual growth-promoting components in one formulation. These components have previously been reported individually but not in combination. The specific combinations Applicants have found to be particularly important are: A mixture of trace elements with growth factor (GF), insulin, transferrin, bFGF, fibronectin, vitamin B12, biotin and hydrocortisone. Inclusion of the protein, fetuin reduces the lag phase and increases growth rates. Fibronectin and growth factor are required for cell attachment prior to growth. The proteins may be of animal origin or expressed as recombinant proteins. The use of non-animal sources is preferred because this reduces the risk of contamination.

Insulin may be substituted by insulin growth factor (IGF) at a concentration range of 1-100 ng/ml. Transferrin may be substituted by an alternative iron delivery system to the cells [Mercalfe et al., 1994].

The AD-SFM is chemically defined and has a low content of proteins and has a lower protein content than serum. The lower protein content ensures ease of product purification that is a problem with the prior art serum containing media. Further without serum standard quality control would ensure that there would be no batch to batch variation as is seen for serum containing media. Further, without serum the potential vector for disease is missing. The composition of the medium is of chemicals which are generally available and does not require the 97 components of the prior art. Additionally, the medium

of the present invention can be used with several microcarriers as well as differing plastics.

The above discussion provides a factual basis for the use of serum-free medium for growing anchorage-dependent mammalian cells. The methods used with and the utility of the present invention can be shown by the following non-limiting examples and accompanying figures.

## EXAMPLE

This Example presents data on the development of a novel serum-free formulation for the growth of Vero cells in static and agitated microcarrier cultures.

### MATERIALS AND METHODS

**Cell line:** African green monkey (Vero) cells were obtained from the American Type Culture Collection (ATCC) CCL 81. The cells were maintained in T-flasks in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% iron-enriched calf serum (Gibco).

**Cultures:** Stock cultures were maintained in T-flasks and passaged every 72-96 hours by trypsinization followed by inoculation into fresh medium (1:10 dilution). Experimental cultures were established in T-25 flasks by inoculation at $10^5$ cells/ml in 10 ml medium. Microcarrier cultures (100 ml) were established in glass spinner flasks (Bellco) with Cytodex-1 (Pharmacia) or Cultispher-G (Hyclone) microcarriers. The Cytodex cultures were stirred continuously at 40 rpm. The Cultispher cultures were stirred intermittently for 24 hours then continuously at 40 rpm for 72 hours at which point the stirring speed was increased to 60 rpm. The stirring regimen of the microcarrier cultures was based upon a previous study [Ng et al, 1996]. All cultures were incubated at 37°C with a 10% $CO_2$ overlay.

**Serum-free medium:** The serum-free medium for Vero cells (VSFM) of the present invention was prepared by adding supplements to DMEM as a basal

medium (Table 2). The DMEM contained 25 mM glucose, 4 mM glutamine, 36 mM sodium bicarbonate. All supplements were prepared in stock concentrate solutions (x100). Control commercial serum-free media was obtained as TCM concentrate (Celox) which replaced serum in enriched DMEM. Vero maintenance medium formulations (II and III) were generously provided by Gibco.

**Adaptation of stock cells:** Cells were transferred directly from serum-containing medium (SCM) to VSFM or were gradually adapted. Gradual adaptation involved sub-culturing every 48 hours into a mixture of SCM and VSFM. The ratio of SCM/VSFM was altered from 5:1 to 1:10 in 9 stages prior to inoculation into complete VSFM.

## RESULTS

1. **Growth in static cultures**

The growth profile of Vero cells in T-flasks was determined by daily cell counts from replicate cultures containing either SCM or VSFM (Fig. 1). This showed a maximum cell density of $1.79 \times 10^6$ cells/ml in SCM as opposed to $1.13 \times 10^6$ cells/ml in VSFM. The specific growth rate was slightly lower in VSFM and the lag phase was extended to 24 hours (Table 3). The growth profile of cells in VSFM was maintained for 10 passages but over subsequent passages the maximum cell density decreased, so that after 35 passages the cells grew with a reduced growth rate and attained a maximum density of $<30\%$ of the original value.

Results in these cultures showed that the cells that were adapted gradually from SCM to VSFM did not achieve a significantly higher maximum cell density compared to those sub-cultured directly to the serum-free medium.

Experimental cultures were established in VSFM in which selected components were removed. In all cases, there was a significant decrease in maximum cell density attained. This showed that all of the components contained in VSFM were essential for growth. The elimination of fibronectin

from VSFM caused poor cell attachment and resulted in the formation of cell aggregates ( ~ 25 cells) which were unattached to the substratum.

2. **Growth in microcarrier cultures**

Vero cell growth was determined in microcarrier cultures of Cytodex-1 or Cultispher-G using various serum-free media. Figure 2 shows that cell growth occurred in all media tested. Maximum cell densities were significantly higher in Cultispher-G cultures compared to those of Cytodex-1. The maximum cell densities attained in SCM was significantly higher than any of the serum-free media. Of the serum-free media tested, cell yields were significantly (44%) higher in VSFM compared to the other formulations, which gave progressively lower yields in the order TCM > GibcoIII > GibcoII.

The cell attachment was significantly faster in SCM compared to any of the serum-free media. This accounts for the shorter observed lag phase and also there were fewer free cells observed throughout the culturing period which indicates that the cells were more firmly attached to the microcarriers. Fewer free cells were observed in the VSFM cultures compared to the other serum-free cultures. The characteristics of these cultures are identified in Table 4.

3. **Pre-coating the microcarriers**

In an attempt to determine the factors that would cause an effective attachment of cells on to the microcarriers, it was decided to investigate the effect of pre-coating the microcarriers with potential attachment factors prior to culture. The microcarriers were pre-coated by suspension of the beads in DMEM supplemented with 5% serum or with 5 mg/L fibronectin for 6 hours. The beads were subsequently washed in PBS prior to incorporation into cultures.

Figure 3 shows the effect of pre-coating the microcarrier beads on cell growth in VSFM prepared with or without fibronectin. A low growth rate of cells occurred in the absence of fibronectin or with beads that were not pre-coated. However, the addition of fibronectin to VSFM or pre-coating the beads

with serum containing medium was equally effective in increasing the growth rate in the serum-free cultures which attained a maximum cell density of 65-70% of the values attained in SCM.

The effect of pre-coating beads was compared with cultures containing alternative serum-free media (Table 5). The maximum cell densities are shown for cultures containing pre-coated or uncoated beads using various media formulations. This data shows that by pre-coating Cytodex-1 or Cultispher-G microcarriers with either fibronectin or SCM, the VSFM cultures grown in the absence of dissolved fibronectin attained the same cell densities as those containing fibronectin. Maximum cell yields were substantially (2-4 times) lower in the absence of fibronectin or without pre-coating the beads. However, the effect of pre-coating the beads had a negligible effect in increasing the maximum cell densities of cultures in the presence of the other serum-free formulations tested.

This Example shows that the medium of the present invention can be used for anchorage-dependent growth of cells in stationary T-flask cultures and agitated cultures using 2 microcarrier types. In all these cultures cell yields above $10^6$/ml were attained from inocula of 0.5-1 $\times$ $10^5$ cells/ml in 5 days. The observed doubling times in the growth phase was 26 hours which is normal for Vero cells. The cell yields were significantly greater in VSFM compared to three other serum-free formulations tested. Also, the characteristics of Vero cell growth in VSFM cultures are superior to other published reports of serum-free cultures, which are restricted to clump cultures [Litwin, 1992], to a single surface type [Cinatl et al, 1993] or offer extremely low growth rates [Merten et al, 1994] or have a long lag phase [Zhaolie et al., 1996].

Throughout this application, various publications, are referenced by author and year. Full citations for the publications are listed below. The disclosures of these publications in their entireties are hereby incorporated by reference into this application in order to more fully describe the state of the art to which this invention pertains.

The invention has been described in an illustrative manner, and it is to be understood that the terminology which has been used is intended to be in the nature of words of description rather than of limitation.

Obviously, many modifications and variations of the present invention are possible in light of the above teachings. It is, therefore, to be understood that within the scope of the appended claims, the invention may be practiced otherwise than as specifically described.

**Table 1:** Concentration ranges for AD-SFM

| Medium Component | Final Concentration (moles/ litre or mg/l)) |
|---|---|
| Serine | 1 - 4 x $10^{-3}$ M |
| Sodium pyruvate | 0.1 - 1.0 x $10^{-3}$ M |
| Fetuin | 0 - 1000 mg/l |
| Growth factor | 0 - 2 mg/l. |
| Hydrocortisone | 5 - 200 x $10^{-9}$ M |
| Insulin | 5 - 25 x $10^{-7}$ M |
| Prostaglandin $E_1$ | 2.5 - 15 x $10^{-11}$ M |
| Transferrin | 2 - 10 mg/l. |
| Triiodothyronine | 1 - 20 x $10^{-12}$ M |
| Biotin | 1 - 10 x $10^{-6}$ M |
| Choline Chloride | 2.5 - 7.5 x $10^{-5}$ M |
| Glutathione | 1 - 5 x $10^{-5}$ M |
| 2-Mercaptoethanol | 2.5 - 7.5 x $10^{-5}$ M |
| Vitamin $B_{12}$ | 2.5 - 10 x $10^{-7}$ M |
| $CuSO_4.5H_2O$ | 5 - 20 x $10^{-9}$ M |
| $FeSO_4.7H_2O$ | 1 - 10 x $10^{-6}$ M |
| $ZnSO_4.7H_2O$ | 1 - 10 x $10^{-6}$ M |
| $MnSO_4$ | 0.5 - 2 x $10^{-9}$ M |
| $Na_2SeO_3$ | 0.5 - 2 x $10^{-7}$ M |
| $Na_2SiO_3.5H_2O$ | 2 - 7.5 x $10^{-9}$ M |
| $(NH_4)_6Mo_7O_{24}.4H_2O$ | 0.5 - 2 x $10^{-10}$ M |
| $NH_4VO_3$ | 2.5 - 10 x $10^{-9}$ M |
| $NiCl_2.6H_2O$ | 2.5 - 7.5 x $10^{-10}$ M |
| $SnCl_2.2H_2O$ | 2.5 - 10 x $10^{-10}$ M |
| Fibronectin | 1 - 10 mg/l |

## TABLE 2

The optimal formulation of Vero cell serum-free medium (VSFM) for growth of Vero cells or other anchorage-dependent mammalian cells in tissue culture flasks or or on microcarriers

| Medium Component | Component Weight Added (mg/L) | Final Concentration (moles/ litre) |
|---|---|---|
| Serine | 170 | $2x10^{-3}$ |
| Sodium pyruvate | 55 | $5x10^{-4}$ |
| Fetuin | 500 | MW UNK |
| Fibroblast growth factor | $2.0 \times 10^{-3}$ | MW UNK |
| Hydrocortisone | $2.0 \times 10^{-2}$ | $5.5x10^{-8}$ |
| Insulin | 5.0 | $9x10^{-7}$ |
| Prostaglandin $E_1$ | $2.5 \times 10^{-5}$ | $7.2x10^{-11}$ |
| Transferrin | 5.0 | MW UNK. |
| Triiodothyronine | $3.5 \times 10^{-6}$ | $5.2x10^{-12}$ |
| Biotin | 1.0 | $4.1x10^{-6}$ |
| Choline Chloride | 6.0 | $4.3x10^{-5}$ |
| Glutathione | 6.0 | $2x10^{-5}$ |
| 2 -Mercaptoethanol | 3.9 | $5x10^{-5}$ |
| Vitamin $B_{12}$ | 0.4 | $3x10^{-7}$ |
| $CuSO_4.5H_2O$ | $2.5 \times 10^{-3}$ | $1x10^{-8}$ |
| $FeSO_4.7H_2O$ | 1.0 | $3.6x10^{-6}$ |
| $ZnSO_4.7H_2O$ | 1.0 | $3.6x10^{-6}$ |
| $MnSO_4$ | $1.7 \times 10^{-4}$ | $1x10^{-9}$ |
| $Na_2SeO_3$ | $1.7 \times 10^{-2}$ | $1x10^{-7}$ |
| $Na_2SiO_3.5H_2O$ | $1.4 \times 10^{-3}$ | $5x10^{-9}$ |
| $(NH_4)_6Mo_7O_{24}.4H_2O$ | $1.2 \times 10^{-4}$ | $1x10^{-10}$ |
| $NH_4VO_3$ | $5.9 \times 10^{-4}$ | $5x10^{-9}$ |
| $NiCl_2.6H_2O$ | $1.2 \times 10^{-4}$ | $5x10^{-10}$ |
| $SnCl_2.2H_2O$ | $1.1 \times 10^{-4}$ | $5x10^{-10}$ |
| Fibronectin | 5.0 | MW UNK. |

MW UNK. - Molecular weight unknown.

TABLE 3

The effect of the medium formulation on Vero cell growth rate, lag phase and attachment times in T25 culture flasks.

| Effect | Time Taken for Particular Effect | |
|---|---|---|
| | DMEM + 5.0% v/v sCS | VSFM |
| Cell Attachment Time | 1.50 ± 0.50 h | 6.00 ± 1.00 h |
| Cell Flattening Time | 6.00 ± 1.00 h | NF |
| Lag Phase | 6.00 ± 0.50 h | 24.00 ± 2.00 h |
| Cell Growth Rate | 0.036 ± 0.003 $h^{-1}$ | 0.027 ± 0.003 $h^{-1}$ |

NF - Never became flattened on the flask surface.

The cells were inoculated at $0.10 \times 10^6$ cells/ml in 10 ml of VSFM and DMEM + 5.0% v/v sCS and grown with a 10% v/v $CO_2$ in air overlay. Once culture flask was sacrificed daily for cell counts by the trypan blue method. The values are means from cultures of cells preadapted either directly of gradually into VSFM and are n=4.

The effect of the **serum-free medium formulation** and DMEM + 5.0% v/v sCS on **cell growth rate**, **lag phase** and **attachment times** in 100 mL spinner flask cultures of **1.00 g/L Cytodex-1** and **1.70 g/L Cultispher-G**. The cells were preadapted either directly or gradually (>6 passages) from DMEM + 5.0% v/v sCS into VSFM, Gibco Maintenance Medium II and III and TCM supplemented DMEM formulations. The cells were inoculated at 0.05 x $10^6$ cells/mL. The flasks were readjusted to pH 7.1 daily after 72 h in culture with 1.00 M HCl or 2.00 M NaOH. The values are means of n=2.

TABLE 4

| Cultures Effect | Time Taken for Particular Effect | | | | |
|---|---|---|---|---|---|
| | DMEM + 5.0% v/v sCS | VSFM | Gibco II | Gibco III | TCM |
| Cytodex-1 | | | | | |
| Cell Attachment Time | 1.00 ± 0.25 h | 2.50 ± 1.00 h | 2.50 ± 1.00 h | 2.50 ± 1.00 h | 2.00 ± 1.00 h |
| Cell Flattening Time | 5.00 ± 1.00 h | 12.00 ± 1.00 h# | 12.00 ± 1.00 h# | 12.00 ± 1.00 h# | 12.00 ± 1.00 h# |
| Lag Phase | 4.00 ± 1.00 h | 21.00 ± 2.00 h | 24.00 ± 2.00 h | 24.00 ± 2.00 h | 21.00 ± 2.00 h |
| Cell Growth Rate | 0.036 ± 0.003 $h^{-1}$ | 0.028 ± 0.003 $h^{-1}$ | 0.024 ± 0.002 $h^{-1}$ | 0.024 ± 0.002 $h^{-1}$ | 0.025 ± 0.002 $h^{-1}$ |
| Cultispher-G | | | | | |
| Cell Attachment Time | 9.00 ± 3.00 h | 15.00 ± 1.00 h | 15.00 ± 1.00 h | 18.00 ± 2.00 h | 15.00 ± 1.00 h |
| Cell Flattening Time | UNK | UNK | UNK | UNK | UNK |
| Lag Phase | 9.00 ± 2.00 h | 21.00 ± 2.00 h | 24.00 ± 2.00 h | 24.00 ± 2.00 h | 21.00 ± 2.00 h |
| Cell Growth Rate | 0.035 ± 0.003 $h^{-1}$ | 0.027 ± 0.003 $h^{-1}$ | 0.024 ± 0.002 $h^{-1}$ | 0.025 ± 0.002 $h^{-1}$ | 0.026 ± 0.002 $h^{-1}$ |

UNK - Unknown as impossible to visualise.
\# - Cells never fully flattened but this was the time at which they became flattest.

TABLE 5

The effects of precoating Cytodex-1 (A) and Cultispher-G (B) beads with either DMEM containing sCS or bovine fibronectin on the maximum Vero cell yields in serum-free medium.

A) - Cytodex-1.

| Serum-Free Medium Formulation | Maximum Cell Concentration (x $10^6$/mL) | | |
|---|---|---|---|
| | Precoated | | Uncoated |
| | DMEM + 5.0% v/v sCS | 5.0 mg/L Fibronectin | |
| VSFM – Fibronectin | 1.17 ± 0.07* | 1.16 ± 0.07 | 0.31 ± 0.03 |
| VSFM + Fibronectin | ND | ND | 1.06 ± 0.07 |
| Gibco II | 0.81 ± 0.06 | 0.78 ± 0.05 | 0.68 ± 0.04 |
| Gibco III | 0.76 ± 0.06 | 0.76 ± 0.05 | 0.75 ± 0.05 |
| TCM-DMEM + Serine and Pyruvate | 0.88 ± 0.07 | 0.85 ± 0.06 | 0.77 ± 0.06 |

B) - Cultispher-G.

| Serum-Free Medium | Maximum Cell Concentration (x $10^6$/mL) | | |
|---|---|---|---|
| | Precoated | | Uncoated |
| | DMEM + 5.0% v/v sCS | 5.0 mg/L Fibronectin | |
| VSFM – Fibronectin | 1.47 ± 0.10* | 1.29 ± 0.08 | 0.66 ± 0.04 |
| VSFM + Fibronectin | ND | ND | 1.27 ± 0.08 |
| Gibco II | 0.90 ± 0.07 | 0.80 ± 0.05 | 0.76 ± 0.05 |
| Gibco III | 0.96 ± 0.06 | 0.87 ± 0.05 | 0.83 ± 0.06 |
| TCM-DMEM + Serine and Pyruvate | 0.94 ± 0.07 | 0.89 ± 0.06 | 0.77 ± 0.06 |

* - Highest cell yield.
ND - Value not determined.

Preadapted cells were inoculated at $0.05 \times 10^6$ cells/ml on 1.00 g/L Cytodex-1 or 1.70 g/L Cultispher-G in 100 ml spinner flasks. The microcarriers were precoated (18 hours) at 20°C. The cells were grown in VSFM, Gibco Vero cell maintenance media II or III or in Celox TCM supplemented, enriched DMEM. The flasks were readjusted daily after 72 hours in culture to pH 7.1 with 1.00 M HCl or 2.00 M NaON. The values are means of n=2.

## REFERENCES

Bradshaw, et al., 1983. The growth requirements of BHK-21 cells in serum-free culture. J. Cell Physiol. 114: 215-221.

Butler, 1992. Supplements to basal media. In Cell Culture: Labfax (Butler, M. and Dawson, M. ed.) pp. 107-133 publ. Bios Sci., Oxford.

Butler and Huzel, 1995. The effect of fatty acids on hybridoma cell growth and antibody productivity in serum-free cultures. Journal of Biotechnology. 39: 165-173.

Cinatl, et al., 1993. Protein-free culture of Vero cells: A substrate for replication of human pathogenic viruses. Cell Biol. Int. 17, 885-895.

Cinatl, et al., 1992. Polyvinyl formal surface promotes continuous growth of Vero cells in protein-free medium. Biologicals 20, 59-65.

Crespi, et al., 1981. Microcarrier culture: Applications in biological production and control. Biotechnol. Bioeng. 23: 2673-2689.

Forestall, et al., 1992. Development of the optimal conditions for microcarrier cultures. Biotechnol. Bioeng. 39, 305-313.

Litwin, 1992. The growth of Vero cells in suspension as cell aggregates in serum-free media. Cytotechnology 10, 169-174.

Merten, et al., 1994. Evaluation of the new serum-free medium (MDSS2) for the production of different biologicals: Use of various cell line. Cytotechnology 14, 47-49.

Metcalf, et al., 1994. The use of 2-hydroxy-2,4,6-cycloheptarin-1-one (Tropolone) as a replacement for transferrin. *In* Animal Cell Technology: products of today, prospects for tomorrow (Spier, Griffiths and Berthold, eds.) European Society for Animal Cell Technology 12:88-90.

Montagnon, et al., 1984. Thousand litre scale microcarrier culture of Vero cells for killed polio virus vaccine. Develop. Biol. Stand. 55, 37-42.

Ng, et al., 1996. Optimisation of physical parameters for cell attachment and growth on macroporous microcarriers. Biotech. Bioeng. in press.

Sayer, et al., 1987. The attachment of MDCK cells in three types of microcarriers in different serum-free media. In Modern Approaches to Animal Cell Technology (eds. Spier, R.E. and Griffiths, J.B.). ESACT 8: 264-279.

Taub, et al., 1979. Growth of Madin-Darby canine kidney epithelial cell (MDCK) line in hormone-supplemented serum-free medium. Proc. Natl. Acad. Sci. 76: 3338-3342.

Van Wezel, 1985. Monolayer growth systems: homogeneous unit processes. In: Spier,R.E., Griffiths, J.B. (eds.) Animal Cell Biotechnology vol 1, pp. 265-282. Academic Press.

Zhaolie, et al., 1996. A novel serum-free medium for the cultivation of Vero cells on microcarriers. Biotech. Tech. 10, 449-452.

## CLAIMS

What is claimed is:

1. A serum-free medium for growing anchorage-dependent mammalian cells in tissue culture including a basal medium supplemented with the components at the concentration range as set forth in Table 1.

2. The serum-free medium as set forth in claim 1 wherein the fibronectin is added to the medium immediately prior to use.

3. The serum-free medium as set forth in claim 1 wherein the tissue culture is stationary.

4. The serum-free medium as set forth in claim 1 wherein the tissue culture is agitated using microcarriers.

5. The serum-free medium as set forth in claim 3 wherein the fibronectin is used to coat tissue culture flasks in which the cells are cultured.

6. The serum-free medium as set forth in claim 4 wherein the fibronectin is used to coat the microcarriers.

7. The serum-free medium as set forth in claim 1 wherein the anchorage-dependent mammalian cells are MRC-5, WI-38, FS-4 (human fibroblasts), Vero (African green monkey), BHK (Baby hamster kidney) and MDCK (Madin Darby canine kidney) and primary cells obtained directly from mammalian tissue.

8. The serum-free medium as set forth in claim 7 wherein the anchorage-dependent mammalian cells are Vero cells.

9. The serum-free medium as set forth in claim 8 wherein the component concentrations are as set forth in Table 2.

10. A serum-free medium for growing anchorage-dependent Vero cells in tissue culture including a basal medium enriched with the components at the concentration range as set forth in Table 2.

11. The serum-free medium as set forth in claim 10 wherein the fibronectin is added to the medium immediately prior to use.

12. A method of culturing anchorage-dependent mammalian cells in tissue culture including the step of

culturing in a basal medium supplemented with the components at the concentration range as set forth in Table 1.

13. The method of culturing as set forth in claim 12 including the step of adding the fibronectin to the medium immediately prior to use.

14. The method of culturing as set forth in claim 12 wherein the tissue culture is stationary.

15. The method of culturing as set forth in claim 12 wherein the tissue culture is agitated using microcarriers.

16. The method of culturing as set forth in claim 14 including the step of coating the tissue culture flasks in which the cells are cultured with fibronectin prior to use.

17. The method of culturing as set forth in claim 15 including the step of coating the microcarriers with which the cells are cultured with fibronectin prior to use.

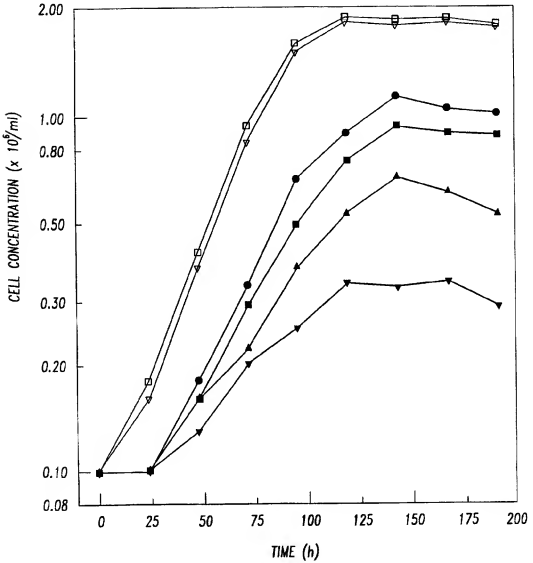
18. The method of culturing as set forth in claim 12 wherein the anchorage-dependent mammalian cells are MRC-5, WI-38, FS-4 (human fibroblasts), Vero (African green monkey), BHK (Baby hamster kidney) and MDCK (Madin Darby canine kidney) and primary cells obtained directly from mammalian tissue.

19. The method of culturing as set forth in claim 18 wherein the anchorage-dependent mammalian cells are Vero cells.

20. The serum-free medium as set forth in claim 19 wherein the component concentrations are as set forth in Table 2.

21. A method for growing anchorage-dependent Vero cells in tissue culture including the step of

culturing in a basal medium supplemented with the components and concentration range as set forth in Table 2.


2.00
1.00
0.80
0.50
0.30
0.20
0.10
0.08
CELL CONCENTRATION (x 10⁶/ml)
0
25
50
75
100
125
150
175
200
TIME (h)

Fig–1

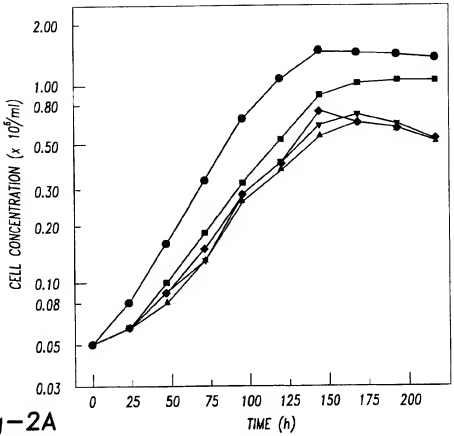

CELL CONCENTRATION (x 10⁶/ml)
2.00
1.00
0.80
0.50
0.30
0.20
0.10
0.08
0.05
0.03
0
25
50
75
100
125
150
175
200
TIME (h)

Fig-2A

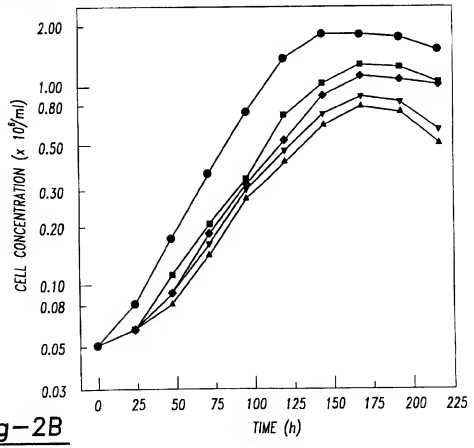

CELL CONCENTRATION (x 10⁶/ml)
2.00
1.00
0.80
0.50
0.30
0.20
0.10
0.08
0.05
0.03
0
25
50
75
100
125
150
175
200
225
TIME (h)

Fig-2B

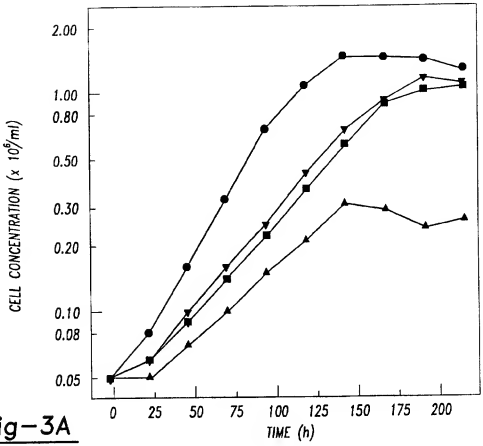

2.00
1.00
0.80
0.50
0.30
0.20
0.10
0.08
0.05
CELL CONCENTRATION (x 10⁶/ml)
0
25
50
75
100
125
150
175
200
TIME (h)

Fig-3A

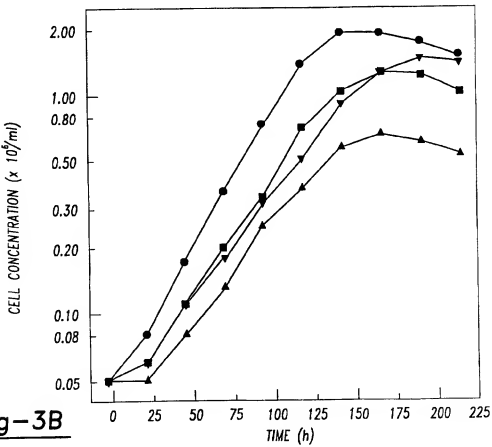

2.00
1.00
0.80
0.50
0.30
0.20
0.10
0.08
0.05
CELL CONCENTRATION (x 10⁶/ml)
0
25
50
75
100
125
150
175
200
225
TIME (h)

Fig-3B

# INTERNATIONAL SEARCH REPORT

International application No.
PCT/US97/13079

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :C12N 5/00, 5/02, 11/00

US CL :435/385, 174, 366, 389, 395, 402, 403, 404, 405

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/385, 174, 366, 389, 395, 402, 403, 404, 405

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS

search terms: serum free medium, microcarrier, anchorage-dependent, fibronectin, Vero cells

C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| Y | US 5,326,699 A (TORISHIMA et al) 05 July 1994, entire document. | 1-21 |
| Y | US 4,560,655 A (BAKER) 24 December 1985, entire document. | 1-21 |
| Y | US 4,786,599 A (CHESSEBEUF et al) 22 November 1988, entire document. | 1-21 |
| Y | US 5,405,772 A (PONTING) 11 April 1995, entire document. | 1-21 |
| Y | US 5,330,911 A (HUBBELL et al) 19 July 1994, entire document. | 4, 6, 15, 17 |
| Y | US 5,147,854 A (NEWMAN) 15 September 1992, entire document. | 5, 16 |

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

| | | | |
|---|---|---|---|
| * | Special categories of cited documents: | "T" | later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention |
| "A" | document defining the general state of the art which is not considered to be of particular relevance | "X" | document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone |
| "E" | earlier document published on or after the international filing date | "Y" | document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art |
| "L" | document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) | "&" | document member of the same patent family |
| "O" | document referring to an oral disclosure, use, exhibition or other means | | |
| "P" | document published prior to the international filing date but later than the priority date claimed | | |

| | |
|---|---|
| Date of the actual completion of the international search<br>18 SEPTEMBER 1997 | Date of mailing of the international search report<br>12 NOV 1997 |
| Name and mailing address of the ISA/US<br>Commissioner of Patents and Trademarks<br>Box PCT<br>Washington, D.C. 20231<br>Facsimile No. (703) 305-3230 | Authorized officer<br>DAVID M. NAFF<br>Telephone No. (703) 308-0196 |

Form PCT/ISA/210 (second sheet)(July 1992)*